Journal of Food Protection, Vol. 75, No. 8, 2012, Pages 1447–1452
doi:10.4315/0362-028X.JFP-12-098

Multilocus Genetic Characterization of Two Ant Vectors (Group II ''Dirty 22'' Species) Known To Contaminate Food and Food Products and Spread Foodborne Pathogens†

IRSHAD M. SULAIMAN,[1]* MICKEY ANDERSON,[1] DAVID H. OI,[2] STEVEN SIMPSON,[1] AND KHALIL KERDAHI[1]

[1]U.S. Food and Drug Administration, Southeast Regional Laboratory, 60 Eighth Street, Atlanta, Georgia 30309; and [2]U.S. Department of Agriculture, Center for Medical, Agricultural, and Veterinary Entomology, 1600 S.W. 23rd Drive, Gainesville, Florida 32608, USA

MS 12-098: Received 27 February 2012/Accepted 26 March 2012

ABSTRACT

The U.S. Food and Drug Administration utilizes the presence of filth and extraneous materials as one of the criteria for implementing regulatory actions and assessing adulteration of food products of public health importance. Twenty-two prevalent pest species (also known as the ''Dirty 22'' species) have been considered by this agency as possible vehicles for the spread of foodborne diseases, and the presence of these species is considered an indicator of unsanitary conditions in food processing and storage facilities. In a previous study, we further categorized the Dirty 22 species into four groups: group I includes four cockroach species, group II includes two ant species, group III includes 12 fly species, and group IV includes four rodent species. Here, we describe the development of three nested PCR primer sets and multilocus genetic characterization by amplifying the small subunit rRNA, elongation factor 1-alpha, and wingless (WNT-1) genes of group II Dirty 22 ant species *Monomorium pharaonis* and *Solenopsis molesta*. These novel group II Dirty 22 species-specific nested PCR primer sets can be used when the specimens cannot be identified using conventional microscopic methods. These newly developed assays will provide correct identification of group II Dirty 22 ant species, and the information can be used in the control of foodborne pathogens.

The primary mission of the U.S. Food and Drug Administration (FDA) is to enforce the Food, Drug, and Cosmetic Act *(46)*. This act forbids the distribution of adulterated food or food products, which includes those products that are defective, unsafe, and filthy or are produced under unsanitary conditions. The filth and other extraneous materials are defined as adulterants under section 402 of the Act, and the FDA implements regulatory action criteria for filth and extraneous materials to assess the adulteration of food products. To accomplish this difficult task, a series of reviews have been published to provide additional guidelines, which include a list of common food pest species (Table 1). These species are also called the ''Dirty 22'' species, and are deemed by the FDA to be a contributory factor for the spread of foodborne diseases *(2, 23–26)*.

In a recent study, we further categorized the Dirty 22 species into four groups: group I includes four cockroach species, group II includes two ant species, group III includes 12 fly species, and group IV includes four rodent species *(37)*. We also described the development and validation of a small subunit (SSU) rRNA gene–based PCR plus restriction fragment length polymorphism protocol that can be used to detect and differentiate the four group I Dirty 22 species. In this follow-up to that previous study, we developed three degenerate nested PCR primer sets based on PCR amplification and nucleotide sequence characterizations of the SSU rRNA, elongation factor 1-alpha (EF-1α), and wingless (WNT-1) genes of the two group II Dirty 22 ant species: *Monomorium pharaonis* (Pharaoh ant) and *Solenopsis molesta* (Thief ant). These ant species are two of the most common household pests of public health importance responsible for contaminating food and spreading foodborne pathogens. All three of these novel PCR assays can differentiate the group II Dirty 22 ant species from other species of food pests, which will further the FDA's mission of keeping the food supply chain safe and preventing foodborne outbreaks.

MATERIALS AND METHODS

Group II Dirty 22 species and DNA extraction. Genomic DNA was extracted from the two ant species belonging to group II of the Dirty 22 species. In most cases, a single specimen (preserved in 70% ethanol) was homogenized using a clean and autoclaved mortar and pestle and liquid nitrogen. The homogenate was purified with the DNeasy Blood & Tissue DNA Extraction Kit (QIAGEN, Valencia, CA) following manufacturer's protocol and

* Author for correspondence. Tel: 404-575-1523; Fax: 404-253-1210; E-mail: irshad.sulaiman@fda.hhs.gov.

† The findings and conclusions in this article are those of the authors and do not necessarily represent the views or official position of the U.S. Food and Drug Administration (FDA) or U.S. Department of Agriculture (USDA). The names of vendors or manufacturers are provided as examples of available product sources; inclusion does not imply endorsement of the vendors, manufacturers, or products by the FDA, USDA, or U.S. Department of Health and Human Services.

TABLE 1. *List of Dirty 22 species as examples of pests that can contribute to the spread of foodborne pathogens*[a]

Species no.	Common name	Scientific name
Group I	Cockroaches	
1	German cockroach	*Blattella germanica* (L.) (Dicityoptera: Blattellidae)
2	Brownbanded cockroach	*Supella longipalpa* (Fabriculus) (Dictyoptera: Blattellidae)
3	Oriental cockroach	*Blatta orientalis* L. (Dictyoptera: Blattidae)
4	American cockroach	*Periplaneta americana* (L.) (Dictyoptera: Blattidae)
Group II	Ants	
5	Pharaoh ant	*Monomorium pharaonis* (L.) (Hymenoptera: Formicidae)
6	Thief ant	*Solenopsis molesta* (Say) (Hymenoptera: Formicidae)
Group III	Flies	
7	House fly	*Musca domestica* L. (Diptera: Muscidae)
8	Stable fly	*Stomoxys calcitrans* (L.) (Diptera: Muscidae)
9	Little house fly	*Fannia canicularis* (L.) (Diptera: Muscidae)
10	Latrine fly	*F. scalaris* (Fabricius) (Diptera: Muscidae)
11	Cosmopolitan blue bottle fly	*Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae)
12	Holarctic blue bottle fly	*C. vomitoria* (L.) (Diptera: Calliphoridae)
13	Oriental latrine fly	*Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae)
14	Secondary screwworm	*Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae)
15	Blue bottle fly	*Cynomyopsis cadaverina* Robineau-Desvoidy (Diptera: Calliphoridae)
16	Green bottle fly	*Phaenicia sericata* (Meigen) (Diptera: Calliphoridae)
17	Black blow fly	*Phormia regina* (Meigen) (Diptera: Calliphoridae)
18	Redtailed flesh fly	*Sarcophaga haemorrhoidalis* (Fallén) (Diptera: Sarcophagidae)
Group IV	Rodents	
19	House mouse	*Mus musculus* (Mammalia: Muridae)
20	Polynesian rat	*Rattus exulans* (Mammalia: Muridae)
21	Norway rat	*R. norvegicus* (Mammalia: Muridae)
22	Roof rat	*R. rattus* (Mammalia: Muridae)

[a] This list was originally published by Olsen et al. *(26)* and further categorized into four groups (I through IV) by Sulaiman et al. *(37).*

recommendations for purification of total DNA from insects. The concentration of purified DNA samples was measured at 260 nm absorbance using a NanoDrop-1000 spectrophotometer (NanoDrop Technology, Rockland, DE), and the samples were stored at −20°C until used.

Nested PCR amplification. To amplify the fragments of the three SSU rRNA, EF-1α, and WNT-1 genes from *M. pharaonis* and *S. molesta,* three novel nested PCR protocols were developed using primers complementary to the conserved regions of the nucleotide sequences of the three genes as published in GenBank: SSU rRNA (GenBank DQ353430, EF012934, and EF012886), EF-1α (GenBank EU204326, EU204417, EU204568, EF013348, and EF013510), and WNT-1 (GenBank EU204173 and EF013770). For the primary and secondary PCR amplification of the SSU rRNA, EF-1α, and WNT-1 genes, six pairs of degenerate primer sets were designed (Table 2). For the primary PCR, the 50-μl reaction consisted of 25 μl of HotStarTaq Master Mix (QIAGEN) and 25 μl of a solution containing 200 nM concentrations of each primer, 1.5 mM additional $MgCl_2$ (Promega, Madison, WI), and template DNA (50 ng) diluted in PCR grade water. The QIAGEN HotStarTaq Master Mix is a premixed solution containing HotStarTaq DNA polymerase, PCR buffer, 200 μM concentrations of deoxynucleoside triphosphates, and a final concentration of 1.5 mM $MgCl_2$. The reactions were run for 35 cycles (each cycle was 94°C for 45 s, 55°C for 45 s, and 72°C for 60 s) in a GeneAmp PCR 9700 thermocycler (Applied Biosystems, Foster City, CA), with an initial hot start (94°C for 15 min) and a final extension (72°C for 10 min). For the secondary PCR, 2.5 ml of primary reaction was used as the template with the nested forward and reverse primers. The conditions for the secondary reaction were identical to those of the primary reaction. The PCR products were analyzed by agarose gel electrophoresis, and bands were visualized after ethidium bromide staining.

Nucleotide sequencing and data analyses. The SSU rRNA, EF-1α, and WNT-1 gene-based nested PCR amplified products of the two ant species (*M. pharaonis* and *S. molesta*) were sequenced using Big-Dye 3.1 dye chemistry and 3500 XL automated DNA sequencers (Applied Biosystems). Nucleotide sequence accuracy was confirmed by two-directional sequencing. For each species, overlapping primer sets were designed and utilized to obtain complete coverage of the amplified fragment. Multiple alignments of the nucleotide sequences were constructed using the BioEdit, ClustalW, and Geneious programs with manual adjustments.

Nucleotide sequence accession numbers. The nucleotide sequences generated for the SSU rRNA, EF-1α, and WNT-1 genes of *M. pharaonis* and *S. molesta* were deposited in GenBank under accession no. JQ695802 to JQ695807.

RESULTS

Twenty specimens belonging to the two group II Dirty 22 ant species (10 specimens each of *M. pharaonis* and *S. molesta*) were used in the study. The three unique degenerate nested PCR primer sets designed based on the SSU rRNA, EF-1α, and WNT-1 genes were used to amplify DNA from these ants. These primer sets were further tested for sensitivity and robustness by performing PCR amplifi-

TABLE 2. *Degenerate nested primer sets designed in this study and used for multilocus sequence characterization*

Gene	Primer	Sequence (5′–3′)
SSU rRNA	IMS-GR2-SSU-F1	CATGCATGTCTCAGTGC
SSU rRNA	IMS-GR2-SSU-R1	CGCGRGGCTAAGCCCACCGGTAG
SSU rRNA	IMS-GR2-SSU-F2	TGTCTCAGTGCATGCCGAATTAAG
SSU rRNA	IMS-GR2-SSU-R2	TAAGCCCACCGGTAGGACG
EF-1	IMS-GR2-EF-F1	TGGAARTTYGAAACVTC
EF-1	IMS-GR2-EF-R1	GCCTTRTCRGTSGGC
EF-1	IMS-GR2-EF-F2	AARTTYGAAACVTCBAARTAC
EF-1	IMS-GR2-EF-R2	TCRGTSGGCYTRGWVGG
WNT-1	IMS-GR2-WNT-F1	CCGAACTTCCGCGTGG
WNT-1	IMS-GR2-WNT-R1	GGTGCAGGAGCACCTCTC
WNT-1	IMS-GR2-WNT-F2	ACTTCCGCGTGGTCGGCGAC
WNT-1	IMS-GR2-WNT-R2	AGCACCTCTCGATTACCGTC

cation at least three times with the genomic DNA of all 10 specimens of each species, followed by nucleotide sequencing of the amplified nested PCR products (Table 2).

Of the 60 nested PCR amplified products of these specimens, 36 were subjected to sequencing to generate the unique nucleotide sequences; 12 sequences each for the SSU rRNA (~700 bp), EF-1α (~500 bp), and WNT-1 (~400 bp) secondary PCR fragments belonging to the six specimens each of *M. pharaonis* and *S. molesta,* respectively (Fig. 1).

The 18S rRNA gene exhibited significant interspecies polymorphism among the 12 specimens of the two ant species sequenced. However, genetic diversity at the intraspecies level was not evident. To date, no SSU rRNA nucleotide sequence has been reported for *M. pharaonis*; thus, the sequences generated in this study are novel and described here for the first time. However, these novel *M. pharaonis* sequences had four-point and five-point differences compared with the two other published species of *Monomorium,* respectively (Table 3). The SSU rRNA sequences generated in this study for *S. molesta* were identical to the published sequence (GenBank EF012934).

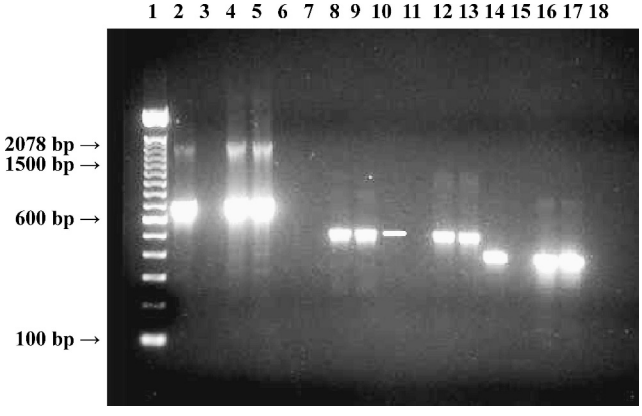

FIGURE 1. *Multilocus PCR amplification of two group II Dirty 22 ant species by nested degenerate PCR primer sets. Lane 1, 100-bp molecular weight marker; lanes 2 through 7, 18S rRNA amplified products; lanes 8 through 13, EF-1a amplified products; lanes 14 through 18, WNT-1 amplified products. Lanes 3, 6, 7, 11, 15, and 18, negative controls; lanes 2, 8 through 10, and 14,* M. pharaonis *PCR amplified products; lanes 4, 5, 12, 13, 16, and 17,* S. molesta *PCR amplified products.*

The EF-1α gene also displayed interspecies variation for these two ant species, but intraspecies genetic diversity was lacking. However, when these sequences were aligned and compared with the published EF-1a nucleotide sequences (GenBank EU204568 and EF013510), one-point mutations were evident in *M. pharaonis* at position 105 (T to C) and in *S. molesta* at position 387 (C to T).

The WNT-1 gene had diversity at both the inter- and intraspecies level for the *M. pharaonis* and *S. molesta* specimens, similar to that exhibited by the 18S rRNA and EF-1α loci for these species. However, the WNT-1 *M. pharaonis* sequences generated completely matched the published sequence (GenBank EU204173), and the *S. molesta* sequence had a two-point mutation (G to A at position 114 and G to T at position 147) when compared with the published sequence (GenBank EF013770).

DISCUSSION

The principal mission of the FDA as a regulatory agency is to prohibit distribution of unsafe and deleterious food, drug, and cosmetic products and to keep the supply chain free of products that can pose a public health risk. This task has become very challenging in recent years. The global production of FDA-regulated goods has exploded over the past decade, and the amount of imported material and ingredients used for production of finished products in U.S. manufacturing facilities has increased phenomenally. Consequently, for efficient regulation several strategies have been considered and developed, including the estimation of hazardous and nonhazardous filth and extraneous materials responsible for the adulteration of food products *(2, 23–26).* The scientific basis for making a regulatory decision should also be considered when analyzing a food sample for common food pests that can contribute to the spread of foodborne pathogens. The first published list of food pest species included 22 insect and rodent species, (four cockroaches, two ants, 12 flies, and four rodents *(26)*), and these species have been called the Dirty 22 by FDA entomologists. These Dirty 22 species have been further

TABLE 3. *Differences between the novel 18S rRNA sequence of* M. pharaonis *generated in this study and the two published* Monomorium *sequences*

M. pharaonis[a]	*M. destructor*[b]	*M. ergatogyna*[c]	Position (nt)
	Four-point mutations		
A	C	A	82
C	G	C	191
G	A	G	192
C	T	C	655
	Five-point mutations		
T	T	A	195
C	C	T	196
C	C	T	198
G	G	C	200
T	T	G	218

[a] Representative sequences determined in this study have been deposited in the GenBank, with accession no. JQ695802 to JQ695807.
[b] From GenBank DQ353430.
[c] From GenBank EF012886.

categorized into four groups based on their taxonomic and molecular characteristics *(37)*.

Molecular genetic characterization (partial and complete gene or genome) of anthroponotic and zoonotic pathogens and their vectors has led to the development of rapid detection methods that include some protocols with high-throughput capability *(1, 14, 15, 17, 20, 42, 43)*. These tools have helped researchers to ascertain the vector species and transmission routes for infectious pathogens and to conduct epidemiologic surveys of public health importance. In recent years, these tools have been extremely useful for controlling several emerging infectious diseases and preventing foodborne and waterborne outbreaks worldwide *(8, 38–41)*. In several human pathogens with very low natural genetic diversity, the multilocus characterizations have been valuable for determining the extent of genetic variation *(19, 31, 32, 38–41)*. To date, molecular characterization of the common food pests including the list of Dirty 22 species, which are known to spread pathogens causing foodborne disease, has been inadequate. Accurate molecular characterization would facilitate the rapid detection and differentiation of these pest species. These novel molecular tools will help the FDA to accomplish one of its most important mandates for food safety programs, i.e., the surveillance of food storage areas, and to conduct molecular epidemiologic investigations of foodborne outbreaks and the involvement of a specific vector species.

Of the nonregulatory housekeeping genes, the SSU RNA and EF-1α genes have been widely exploited for describing genetic polymorphism across eukaryotic organisms, including various ant species *(3, 9, 12, 18, 36)*. The SSU rRNA genes are the most highly conserved regions of the eukaryotic genome, often with several copies, and are thought to evolve at a relatively slower rate *(1, 37)*. The rRNA gene has been most extensively used as a phylogenetic marker for exploring taxonomic and evolutionary relationships among species and has been useful in the development of diagnostic methods for detection and differentiation of species at the molecular level *(22, 44)*. This gene also has been used successfully to assess the genetic relationships among various insect species, including the two group II Dirty 22 ant species *M. pharaonis* and *S. molesta (1, 4–7, 13, 29, 47, 50)*.

The EF-1α is another conserved gene that has been recognized as a useful genetic marker for assessing higher level phylogenetic relationships among insect species; this gene also can occur as single or multiple copies *(10, 11, 30)*. The WNT-1 gene (wingless) also has been widely characterized and used in molecular evolutionary analyses and phylogenetic studies of several insect species *(21, 33)*. Mitochondrial genes such as cytochrome oxidase I, cytochrome oxidase II, and the carbomoylphosphate synthetase domain of CAD (a multienzyme-encoding gene) also have been used as genetic markers for exploring species relationships among insects *(4, 7, 16, 29, 50)*.

Ants are social insects belonging to the family Formicidae and are thought to have evolved from wasp-like ancestors in the mid-Cretaceous period between 110 and 130 million years ago. Of the estimated 22,000 ant species, only 12,500 species have been classified. Ants are found on almost every continent and thrive in most ecosystems, except in Antarctica and a few other inhospitable islands. Ants represent an estimated 15 to 25% of the terrestrial animal biomass. Because of their natural survival skills and social nature, ants have developed commensal, mutualistic, and parasitic relationships with other species during the course of coevolution. Although ants serve several ecological functions valuable to humans, including control of pest populations and soil aeration, some ant species are considered pests *(28, 34, 35, 48)*.

To explore the evolutionary and phylogenetic relationships among various ant species and between ants and other insects at various taxonomic levels, the SSU rRNA, EF-1α, and WNT-1 loci have been widely used as genetic markers *(16, 27, 44, 45, 49)*. While characterizing the 18S rRNA, EF-1α, and WNT-1 loci of several Pharaoh ant and Thief ant specimens, we found that these three genes were very useful for the detection of and differentiation between these two ant species. Analyses of data revealed a significant interspecies genetic polymorphism, and in some cases intraspecies genetic variation also was obvious (Table 3). Our study confirmed that PCR amplification followed by nucleotide sequencing could differentiate between *M. pharaonis* and *S. molesta* specimens. The newly designed degenerate primer sets based on the SSU rRNA, EF-1α, and WNT-1 loci were robust and could amplify successfully at a range of PCR parameters (data not shown).

In the current FDA filth monitoring program, insects and insect fragments are identified by conventional macroscopic and microscopic morphological characteristics described in insect keys *(26, 37)*. However, the features needed for identification often are lost or decayed to the extent that a trained entomologist cannot identify the specimen at the family level, which is a critical for the analysis of an official regulatory sample. In food samples, identification of the food pest (Dirty 22 species) to the

species level is necessary for violations to be documented so corrective action can be taken. The presence of any of the Dirty 22 species in food samples is considered hazardous to human health *(26)*. Thus, a rapid diagnostic method that can detect and differentiate these pests in food and food products is needed to accomplish the mission of the FDA.

In summary, in this study three degenerate nested PCR primers sets were developed based on the SSU rRNA, EF-1α, and WNT-1 genes and used to amplify the genomic DNA of two violative ant species (*M. pharaonis* and *S. molesta*) belonging to group II of the Dirty 22 species. Nucleotide sequencing of the resultant secondary amplified PCR products of all three genes characterized was used to differentiate the two ant species at the molecular level. Thus, this method will be useful when conventional morphological methods fail to identify the specific group II Dirty 22 species in official regulatory food samples, especially when recovery of these pests is a concern. Efforts are underway to characterize the members of group III and group IV Dirty 22 species at the molecular level to elucidate their genetic makeup and develop novel diagnostic tools for their detection and regulation in official food samples.

ACKNOWLEDGMENTS

This study was supported in part by funding from the Division of Field Sciences (DFS) of the FDA. I. M. Sulaiman is the principal investigator of the DFS Challenge Initiative Program grant. The funders had no role in the study design, data collection, data analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. Aransay, A. M., E. Scoulica, B. Chaniotis, and Y. Tselentis. 1999. Typing of sand flies from Greece and Cyprus by DNA polymorphism of 18S rRNA gene. *Insect Mol. Biol.* 8:179–184.
2. Biles, P. V., and G. C. Ziobro. 2000. Regulatory action criteria for filth and other extraneous materials. IV. Visual detection of hair in food. *Regul. Toxicol. Pharmacol.* 32:73–77.
3. Brady, S. G., T. R. Schultz, B. L. Fisher, and P. S. Ward. 2006. Evaluating alternative hypotheses for the early evolution and diversification of ants. *Proc. Natl. Acad. Sci. USA* 103:18172–18177.
4. Bybee, S. M., S. D. Taylor, N. C. Riley, and M. F. Whiting. 2004. A phylogeny of robber flies (Diptera: Asilidae) at the subfamilial level: molecular evidence. *Mol. Phylogenet. Evol.* 30:789–797.
5. Carmean, D., L. S. Kimsey, and M. L. Berbee. 1992. 18S rDNA sequences and the holometabolous insects. *Mol. Phylogenet. Evol.* 1: 270–278.
6. Carreno, R. A., and J. R. Barta. 1998. Small subunit ribosomal RNA genes of tabanids and hippoboscids (Diptera: Brachycera): evolutionary relationships and comparison with other Diptera. *J. Med. Entomol.* 35:1002–1006.
7. Caterino, M. S., S. Cho, and F. A. Sperling. 2000. The current state of insect molecular systematics: a thriving Tower of Babel. *Annu. Rev. Entomol.* 45:1–54.
8. Cliver, D. O. 2010. Early days of food and environmental virology. *Food Environ. Virol.* 2:1–23.
9. Cruickshank, R. H., K. P. Johnson, V. S. Smith, R. J. Adams, D. H. Clayton, and R. D. M. Page. 2001. Phylogenetic analysis of partial sequences of elongation factor 1a identifies major groups of lice (Insecta: Phthiraptera). *Mol. Phylogenet. Evol.* 19:202–215.
10. Danforth, B. N., S. G. Brady, S. D. Sipes, and A. Pearson. 2004. Single-copy nuclear genes recover Cretaceous-age divergences in bees. *Syst. Biol.* 53:309–326.
11. Danforth, B. N., and S. Ji. 1998. Elongation factor-1a occurs as two copies in bees: implications for phylogenetic analysis of EF-1a sequences in insects. *Mol. Biol. Evol.* 15:225–235.
12. Danielson, K. G., J. Pillarisetti, I. R. Cohen, B. Sholehvar, K. Huebner, L. Ng, J. M. Nichollsi, K. S. E. Cheah, and R. V. Iozzo. 1995. Characterization of the complete genomic structure of the human WNT-5A gene, functional analysis of its promoter, chromosomal mapping, and expression in early human embryogenesis. *J. Biol. Chem.* 270:31225–31234.
13. Dittmar, K., M. L. Porter, S. Murray, and M. F. Whiting. 2006. Molecular phylogenetic analysis of nycteribiid and streblid bat flies (Diptera: Brachycera, Calyptratae): implications for host associations and phylogeographic origins. *Mol. Phylogenet. Evol.* 38:155–170.
14. Giribet, G., G. D. Edgecombe, and W. C. Wheeler. 2001. Arthropod phylogeny based on eight molecular loci and morphology. *Nature* 413:157–161.
15. Heinze, J., A. Trindl, B. Seifert, and K. Yamauchi. 2005. Evolution of male morphology in the ant genus *Cardiocondyla*. *Mol. Phylogenet. Evol.* 37:278–288.
16. Jenkins, T. M., R. E. Dean, R. Verkerk, and B. T. Forschler. 2001. Phylogenetic analyses of two mitochondrial genes and one nuclear intron region illuminate European subterranean termite (Isoptera: Rhinotermitidae) gene flow, taxonomy, and introduction dynamics. *Mol. Phylogenet. Evol.* 20: 286–293.
17. Kato, H., A. G. Cáceres, E. A. Gomez, T. Mimori, H. Uezato, J. D. Marco, P. A. Barroso, H. Iwata, and Y. Hashiguchi. 2008. Molecular mass screening to incriminate sand fly vectors of Andean-type cutaneous leishmaniasis in Ecuador and Peru. *Am. J. Trop. Med. Hyg.* 79:719–721.
18. Kjer, K. M. 2004. Aligned 18S and insect phylogeny. *Syst. Biol.* 53: 506–514.
19. Morgan-Rayan, U., A. Fall, L. A. Ward, N. Hijjawi, I. M. Sulaiman, R. Fayer, R. C. A. Thompson, M. Olson, A. Lal, and L. Xiao. 2002. *Cryptosporidium huminis* n. sp. (Apicomplexa: Cryptosporidiidae) from *Homo sapiens*. *J. Eukaryot. Microbiol.* 49:433–440.
20. Mukha, D. V., A. P. Sidorenko, I. V. Lazebnaya, B. M. Wiegmann, and C. Schal. 2000. Analysis of intraspecies polymorphism in the ribosomal DNA cluster of the cockroach *Blattella germanica*. *Insect Mol. Biol.* 9:217–222.
21. Murat, S., C. Hopfen, and A. P. McGregor. 2010. The function and evolution of Wnt genes in arthropods. *Arthropod Struct. Dev.* 39: 446–452.
22. Nirmala, X., V. Hypsa, and M. Zurovec. 2001. Molecular phylogeny of *Calyptratae* (Diptera: Brachycera): the evolution of 18S and 16S ribosomal rDNAs in higher dipterans and their use in phylogenetic inference. *Insect Mol. Biol.* 10:475–485.
23. Olsen, A. R. 1998. Regulatory action criteria for filth and other extraneous materials. I. Review of hard or sharp foreign objects as physical hazards in food. *Regul. Toxicol. Pharmacol.* 28:181–189.
24. Olsen, A. R. 1998. Regulatory action criteria for filth and other extraneous materials. II. Allergenic mites: an emerging food safety issue. *Regul. Toxicol. Pharmacol.* 28:190–198.
25. Olsen, A. R. 1998. Regulatory action criteria for filth and other extraneous materials. III. Review of flies and foodborne enteric disease. *Regul. Toxicol. Pharmacol.* 28:199–211.
26. Olsen, A. R., J. S. Gecan, G. C. Ziobro, and J. R. Bryce. 2001. Regulatory action criteria for filth and other extraneous materials. V. Strategy for evaluating hazardous and nonhazardous filth. *Regul. Toxicol. Pharmacol.* 33:363–392.
27. Petersen, F. T., R. Meier, S. N. Kutty, and B. M. Wiegmann. 2007. The phylogeny and evolution of host choice in the Hippoboscoidea (Diptera) as reconstructed using four molecular markers. *Mol. Phylogenet. Evol.* 45:111–122.
28. Rabeling, C., J. M. Brown, and M. Verhaagh. 2008. Newly discovered sister lineage sheds light on early ant evolution. *Proc. Natl. Acad. Sci. USA* 105:14913–14917.
29. Ratcliffe, S. T., D. W. Webb, R. A. Weinzievr, and H. M. Robertson. 2003. PCR-RFLP identification of Diptera (Calliphoridae, Muscidae and Sarcophagidae)—a generally applicable method. *J. Forensic Sci.* 48:783–785.
30. Regier, J. C., and J. W. Shultz. 2001. Elongation factor-2: a useful gene for arthropod phylogenetics. *Mol. Phylogenet. Evol.* 20:136–148.

31. Ryan, U. M., P. Monis, H. L. Enemark, I. M. Sulaiman, B. Samarasinghe, C. Read, R. Buddle, I. Robertson, L. Zhou, R. C. A. Thompson, and L. Xiao. 2004. *Cryptosporidium suis* n. sp. (Apicomplexa: Cryptosporidiidae) in pigs (*Sus scrofa*). *J. Parasitol.* 4:769–773.
32. Ryan, U. M., L. Xiao, C. Read, I. M. Sulaiman, P. Monis, A. A. Lal, R. Fayer, and I. Pavlasek. 2003. A redescription of *Cryptosporidium galli* Pavlasek, 1999 (Apicomplexa: Cryptosporidiidae) from birds. *J. Parasitol.* 89:809–813.
33. Schubert, M., L. Z. Holland, N. D. Holland, and D. K. Jacobs. 2000. A phylogenetic tree of the Wnt genes based on all available full-length sequences, including five from the cephalochordate *Amphioxus*. *Mol. Biol. Evol.* 17:1896–1903.
34. Schultz, T. R. 2000. In search of ant ancestors. *Proc. Natl. Acad. Sci. USA* 97:14028–14029.
35. Schultz, T. R., and S. G. Brady. 2008. Major evolutionary transitions in ant agriculture. *Proc. Natl. Acad. Sci. USA* 105:5435–5440.
36. Simona, S., B. Schierwatera, and H. Hadrysa. 2010. On the value of elongation factor-1α for reconstructing pterygote insect phylogeny. *Mol. Phylogenet. Evol.* 54:651–656.
37. Sulaiman, I. M., M. Anderson, M. Khristova, K. Tang, N. Sulaiman, E. Phifer, S. Simpson, and K. Kerdahi. 2011. Development of a PCR–restriction fragment length polymorphism protocol for rapid detection and differentiation of four cockroach vectors (group I ''Dirty 22'' species) responsible for food contamination and spreading of foodborne pathogens: public health importance. *J. Food Prot.* 74:1883–1890.
38. Sulaiman, I. M., R. Fayer, C. Bern, R. H. Gilman, P. Schantz, P. Das, A. A. Lal, and L. Xiao. 2003. Triosephosphate isomerase gene characterization and potential zoonotic transmission of multispecies *Giardia duodenalis*. *Emerg. Infect. Dis.* 9:1444–1452.
39. Sulaiman, I. M., R. Fayer, A. A. Lal, J. M. Trout, F. W. Schaefer, and L. Xiao. 2003. Molecular characterization of microsporidia indicates that wild mammals harbor host-adapted *Enterocytozoon* spp. as well as human pathogenic *Enterocytozoon bieneusi*. *Appl. Environ. Microbiol.* 69:4495–4501.
40. Sulaiman, I. M., A. A. Lal, and L. Xiao. 2001. A population genetic study of the *Cryptosporidium parvum* human genotype parasites. *J. Eukaryot. Microbiol.* 24S–27S.
41. Sulaiman, I. M., A. A. Lal, and L. Xiao. 2002. Molecular phylogeny and evolutionary relationships of *Cryptosporidium* parasites at the actin locus. *J. Parasitol.* 88:388–394.
42. Sulaiman, I. M., X. Liu, M. Frace, N. Sulaiman, M. Olsen-Rasmussen, E. Neuhaus, P. Rota, and R. M. Wohlhueter. 2006. Evaluation of Affymetrix severe acute respiratory syndrome resequencing GeneChips in characterization of the genomes of two strains of coronavirus infecting humans. *Appl. Environ. Microbiol.* 72:207–211.
43. Sulaiman, I. M., K. Tang, J. Osborne, S. Sammons, and R. M. Wohlhueter. 2007. GeneChip resequencing of smallpox virus genome can identify novel strains: a biodefense application. *J. Clin. Microbiol.* 45:358–363.
44. Svenson, G. J., and M. F. Whiting. 2009. Reconstructing the origins of praying mantises (Dictyoptera, Mantodea): the roles of Gondwanan vicariance and morphological convergence. *Cladistics* 25:468–514.
45. Terry, M. D., and M. F. Whiting. 2005. Mantophasmatodea and phylogeny of the lower neopterous insects. *Cladistics* 21:240–257.
46. U.S. Food and Drug Administration. 2004. Federal Food, Drug, and Cosmetic Act. As amended through December 31, 2004. U.S. Department of Health and Human Services, Government Printing Office, Washington, DC.
47. Van Borm, S., J. Billen, and J. J. Boomsma. 2002. The diversity of microorganisms associated with *Acromyrmex* leafcutter ants. *BMC Evol. Biol.* 2:9.
48. Ward, P. S. 2007. Phylogeny, classification, and species-level taxonomy of ants (Hymenoptera: Formicidae). *Zootaxa* 1668:549–563.
49. Whiting, M. F., S. Bradler, and T. Maxwell. 2003. Loss and recovery of wings in stick insects. *Nature* 421:264–267.
50. Winterton, S. L., B. M. Wiegmann, and E. I. Schlinger. 2007. Phylogeny and Bayesian divergence time estimations of small-headed flies (Diptera: Acroceridae) using multiple molecular markers. *Mol. Phylogenet. Evol.* 43:808–832.